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INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference PL0379 PCT		FOR FURTHER ACTION		See Form PCT/PEA/416
International application No. PCT/GB2004/004573		International filing date (day/month/year) 29.10.2004		Priority date (day/month/year) 31.10.2003
International Patent Classification (IPC) or national classification and IPC C09B23/02, C07D209/24, C07D209/10, G01N33/58				
Applicant AMERSHAM BIOSCIENCES UK LIMITED et al				
<p>1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 6 sheets, including this cover sheet.</p> <p>3. This report is also accompanied by ANNEXES, comprising:</p> <p>a. <input checked="" type="checkbox"/> sent to the applicant and to the International Bureau a total of 6 sheets, as follows:</p> <p><input checked="" type="checkbox"/> sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).</p> <p><input type="checkbox"/> sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in Item 4 of Box No. I and the Supplemental Box.</p> <p>b. <input type="checkbox"/> (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).</p>				
<p>4. This report contains indications relating to the following items:</p> <p><input checked="" type="checkbox"/> Box No. I Basis of the opinion</p> <p><input type="checkbox"/> Box No. II Priority</p> <p><input type="checkbox"/> Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p><input type="checkbox"/> Box No. IV Lack of unity of invention</p> <p><input checked="" type="checkbox"/> Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p><input type="checkbox"/> Box No. VI Certain documents cited</p> <p><input type="checkbox"/> Box No. VII Certain defects in the international application</p> <p><input checked="" type="checkbox"/> Box No. VIII Certain observations on the international application</p>				
Date of submission of the demand 12.05.2005		Date of completion of this report 06.03.2006		
Name and mailing address of the International preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tlx: 31 651 epo nl Fax: +31 70 340 - 3016		Authorized Officer Ginoux, C Telephone No. +31 70 340-2839		



**INTERNATIONAL PRELIMINARY REPORT
ON PATENTABILITY**

International application No.
PCT/GB2004/004573

Box No. I Basis of the report

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.
- ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
- ☐ international search (under Rules 12.3 and 23.1(b))
 - ☐ publication of the international application (under Rule 12.4)
 - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)
2. With regard to the **elements*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

Description, Pages

1-46 as originally filed

Claims, Numbers

1-16 received on 29.08.2005 with letter of 25.08.2005

Drawings, Sheets

1/9-9/9 as originally filed

- ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing
3. ☐ The amendments have resulted in the cancellation of:
- ☐ the description, pages
 - ☐ the claims, Nos.
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):
4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
- ☐ the description, pages
 - ☐ the claims, Nos.
 - ☐ the drawings, sheets/figs
 - ☐ the sequence listing (*specify*):
 - ☐ any table(s) related to sequence listing (*specify*):

* If item 4 applies, some or all of these sheets may be marked "superseded."

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Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	1-16
	No: Claims	
Inventive step (IS)	Yes: Claims	1-16
	No: Claims	
Industrial applicability (IA)	Yes: Claims	1-16
	No: Claims	

2. Citations and explanations (Rule 70.7):

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

**Reasoned statement with regard to novelty, inventive step or industrial applicability;
citations and explanations supporting such statement**

Reference is made to the following document/s/:

- D1: PATENT ABSTRACTS OF JAPAN vol. 018, no. 126 (P-1702), 2 March 1994 (1994-03-02) -& JP 05 313304 A (FUJI PHOTO FILM CO LTD), 26 November 1993 (1993-11-26)
- D2: PATENT ABSTRACTS OF JAPAN vol. 016, no. 511 (P-1441), 21 October 1992 (1992-10-21) & JP 04 186342 A (FUJI PHOTO FILM CO LTD), 3 July 1992 (1992-07-03)
- D3: US 2002/077487 A1 (LEUNG WAI-YEE ET AL) 20 June 2002 (2002-06-20)

As a consequence of the limitations introduced in the definition of the group -E-F in claim 1, where E now represents a spacer group having a chain from 1-20 linked atoms selected from the group consisting of carbon, nitrogen and oxygen atoms and F is a reactive group selected from succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, haloacetamide and phosphoramidite, the documents D1 and D2 which disclose similar compounds in which F represents a carboxy group cannot be regarded as novelty-destroying for the subject-matter of the present application.

The subject-matter of claim 1 is therefore new (Article 33(2) PCT).

Document D3, which is considered to represent the most relevant state of the art in the field of labelling reagents, discloses (see claims, examples) carbocyanine dyes used for labelling a target component in which there is at least one substituted indolinium ring system, where a substituent on the 3-position of the indolinium ring contains a chemically reactive group or a conjugated substance.

From this, the subject-matter of independent claim 1 differs in that at least one of the substituents on the 3-position of the indolinium rings (R^{11} - R^{14}) carries a sulfonic acid or phosphonic acid group, while at least one chemically reactive group -E-F suitable for direct covalent or non-covalent labelling of a target material is present on a different position on the molecule.

The method disclosed in D3 being already characterized by a decrease in the tendency of cyanine dyes to self-associate and therefore by a better fluorescence of the conjugates prepared (see description, page 2, first paragraph), the problem to be solved by the present invention may be regarded as the provision of alternative labelling dyes to the ones disclosed in D3.

The solution to this problem proposed in claim 1 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons: there is no indication in the prior art to modify the dyes used in D3 in such a specific way that a sulfonic acid or phosphonic acid group is carried by an alkylene chain on the 3-position of the indolium ring system(s) while at the same time a substituent carrying a chemically reactive group is moved from such a position to another part of the molecule.

The specific compounds of claim 11 and the indolium compounds of claim 13 are novel in view of the available prior art. They can be seen as intermediates for the compounds of formula (I) of claim 1. Inventive step can thus be acknowledged.

Having regard to the subject-matter of process claim 12 for the preparation of the compounds of claim 1, it is observed that its inventive step is directly dependent from that of the compounds of claim 1.

Claim 15 concerns a method for labelling a target component with compounds of formula (I). In this claim the group F has a broader definition than in claim 1, however the claimed method can still be considered inventive vis-à-vis the method disclosed in D3 for the reasons mentioned above for claim 1.

Claims 2-10, 14 and 16 are dependent on claim 1, 13 and 15 respectively and as such also meet the requirements of the PCT with respect to novelty and inventive step.

Re Item VIII

Dependent claim 7 defines the group F as a target bonding group comprising an affinity tag while dependent claim 10 defines the group -E-F as comprising a carboxypentyl group. These

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(SEPARATE SHEET)**

International application No.

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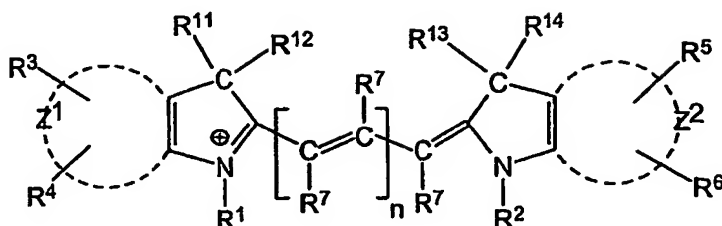
features do not fall within the scope of claim 1 from which these claims are dependent. This inconsistency between the claims leads to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear, Article 6 PCT.

The description is not in conformity with the claims as required by Rule 5.1(a)(iii) PCT.

Claims

1. A compound of formula (I):

5



10

(I)

wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure and groups R^5 and R^6 are attached to the Z^2 ring structure, and $n = 1, 2$ or 3 ;

Z^1 and Z^2 independently represent the carbon atoms necessary to complete a one ring, or two-fused ring aromatic system;

at least one of groups $R^1, R^2, R^3, R^4, R^5, R^6$ and R^7 is the group $-E-F$ where E is a spacer group having a chain from 1–20 linked atoms selected from the group consisting of carbon, nitrogen and oxygen atoms and F is a reactive group selected from succinimidyl ester, sulpho-succinimidyl ester,

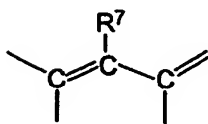
isothiocyanate, maleimide, haloacetamide and phosphoramidite;

one or more of groups R^{11}, R^{12}, R^{13} and R^{14} are independently selected from the group $-(CH_2)_k-W$, where W is sulphonic acid or phosphonic acid and k is an integer from 1 to 10;

when any of groups R^1 and R^2 is not said group $-E-F$, said remaining groups R^1 and R^2 are independently selected from $C_1 - C_6$ alkyl, benzyl either unsubstituted or substituted with sulphonic acid, and the group $-(CH_2)_k-W$, where W and k are hereinbefore defined;

when any of groups R^3, R^4, R^5 and R^6 is not said group $-E-F$, said remaining groups R^3, R^4, R^5 and R^6 are independently selected from hydrogen and sulphonic acid;

when any of groups R^{11}, R^{12}, R^{13} and R^{14} is not said group $-(CH_2)_k-W$, said remaining groups R^{11}, R^{12}, R^{13} and R^{14} are independently $C_1 - C_6$ alkyl; remaining groups R^7 are hydrogen or two of R^7 together with the group,



5 form a hydrocarbon ring system having 5 or 6 atoms.

2. A compound according to claim 1 wherein at least two of R^{11} , R^{12} , R^{13} and R^{14} are independently $-(CH_2)_k-W$ wherein W and k are hereinbefore defined.

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3. A compound according to claim 1 wherein one of groups R^{11} and R^{12} and one of groups R^{13} and R^{14} is the group $-(CH_2)_k-W$ wherein W and k are hereinbefore defined; and remaining groups R^{11} or R^{12} and R^{13} or R^{14} are $C_1 - C_6$ alkyl.

15

4. A compound according to any of claims 1 to 3 wherein W is sulphonic acid.

5. A compound according to any of claims 1 to 3 wherein $-(CH_2)_k-W$ is selected from $-(CH_2)_3-SO_3H$ and $-(CH_2)_4-SO_3H$.

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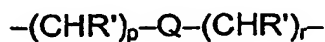
6. A compound according to any of claims 1 to 5 wherein Z^1 and Z^2 are selected from phenyl and naphthyl moieties.

7. A compound according to any of claims 1 to 6 wherein said target bonding group F comprises an affinity tag.

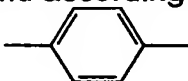
25

8. A compound according to any of claims 1 to 7 wherein said spacer group E is selected from:

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where Q is selected from: $-\text{CHR}'-$, $-\text{NR}'-$, $-\text{O}-$, $-\text{CR}'=\text{CR}'-$, $-\text{Ar}-$, $-\text{C}(\text{O})-\text{NR}'-$ and $-\text{C}(\text{O})-\text{O}-$; R' is hydrogen or $\text{C}_1 - \text{C}_4$ alkyl, p is 0 – 5 and r is 1 – 5.

- 5 9. A compound according to claim 8 wherein Q is selected from: $-\text{CHR}'-$, $-\text{C}(\text{O})-\text{NH}-$ and ; where R' is hereinbefore defined.

10. A compound according to any of claims 1 to 6 wherein said group $-\text{E}-\text{F}$ comprises a carboxypentyl group.

10

11. A compound selected from:

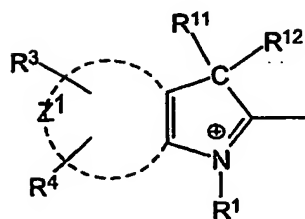
- i) 2-((1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl)-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate;
- 15 ii) 2-((1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl)-3-methyl-1,3-bis(4-sulphobutyl)-3*H*-indolium-5-sulphonate;
- iii) 2-((1*E*,3*E*,5*E*,7*E*)-7-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl)-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate;
- 20 iv) 2-((1*E*,3*E*,5*E*,7*E*)-7-[5-(carboxymethyl)-3-methyl-1,3-bis(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]hepta-1,3,5-trienyl)-1-ethyl-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate; and
- 25 v) 1-benzyl-2-((1*E*,3*E*,5*E*)-5-[1-(5-carboxypentyl)-3-methyl-5-sulpho-3-(4-sulphobutyl)-1,3-dihydro-2*H*-indol-2-ylidene]penta-1,3-dienyl)-3-methyl-3-(4-sulphobutyl)-3*H*-indolium-5-sulphonate.

12. A method for preparing a compound according to any one of claims 1 to 10, the method comprising:

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- a) reacting a first intermediate compound having the formula (A):

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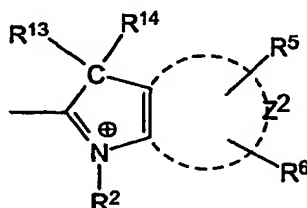


(A)

wherein Z^1 , R^1 , R^3 , R^4 , R^{11} and R^{12} are hereinbefore defined;

- 10 b) a second intermediate compound which may be the same or different from the first intermediate compound and having the formula (B):

15



(B)

wherein Z^2 , R^2 , R^5 , R^6 , R^{13} and R^{14} are hereinbefore defined; and

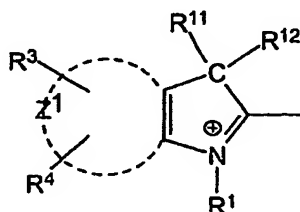
- 20 c) a third compound (C) suitable for forming a linkage between the first and second compounds;

provided that at least one of the groups R^1 , R^2 , R^3 , R^4 , R^5 and R^6 is the group $-E-F$, where E and F are defined as in claim 1; and provided that one or more of groups R^{11} , R^{12} , R^{13} and R^{14} are independently selected from the group

25 $-(CH_2)_k-W$, where W is selected from sulphonic acid and phosphonic acid groups and k is an integer from 1 to 10.

13. A compound of formula:

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wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure, wherein Z^1 is hereinbefore defined;

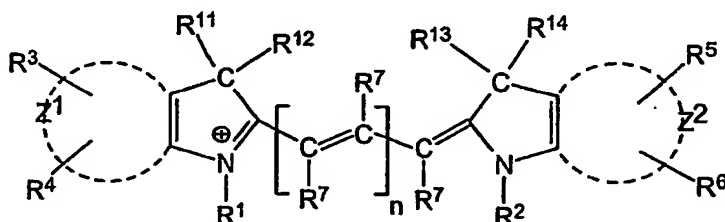
at least one of the groups R^1 , R^3 and R^4 is the group $-E-F$ where E and F are defined as in claim 1; and

at least one of groups R^{11} and R^{12} is the group $-(CH_2)_k-W$, where W is selected from sulphonic acid and phosphonic acid groups and k is an integer from 1 to 10.

14. A compound according to claim 13 wherein $-(CH_2)_k-W$ is selected from $-(CH_2)_3-SO_3H$ and $-(CH_2)_4-SO_3H$.

15. A method for labelling a target component, the method comprising:

i) contacting said component with a compound of formula (I):



(I)

wherein:

groups R^3 and R^4 are attached to the Z^1 ring structure and groups R^5 and R^6 are attached to the Z^2 ring structure, and $n = 1, 2$ or 3 ;

Z^1 and Z^2 independently represent the carbon atoms necessary to complete a one ring, or two-fused ring aromatic system;

at least one of groups R^1 , R^2 , R^3 , R^4 , R^5 , R^6 and R^7 is the group $-E-F$ where E is a single bond or a spacer group having a chain from 1–20 linked atoms selected from the group consisting of carbon, nitrogen and oxygen atoms and

F is a target bonding group;

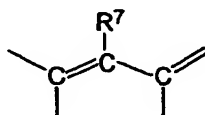
one or more of groups R^{11} , R^{12} , R^{13} and R^{14} are independently selected from the group $-(CH_2)_k-W$, where W is sulphonic acid or phosphonic acid and k is an integer from 1 to 10;

when any of groups R^1 and R^2 is not said group $-E-F$, said remaining groups R^1 and R^2 are independently selected from $C_1 - C_6$ alkyl, benzyl either unsubstituted or substituted with sulphonic acid, and the group $-(CH_2)_k-W$, where W and k are hereinbefore defined;

- 5 when any of groups R^3 , R^4 , R^5 and R^6 is not said group $-E-F$, said remaining groups R^3 , R^4 , R^5 and R^6 are independently selected from hydrogen and sulphonic acid;

when any of groups R^{11} , R^{12} , R^{13} and R^{14} is not said group $-(CH_2)_k-W$, said remaining groups R^{11} , R^{12} , R^{13} and R^{14} are independently $C_1 - C_6$ alkyl;

- 10 remaining groups R^7 are hydrogen or two of R^7 together with the group,



- 15 form a hydrocarbon ring system having 5 or 6 atoms; and
 ii) incubating said fluorescent dye with said component under conditions suitable for binding to and thereby labelling said component.

16. A method according to claim 15 wherein said component is selected
 20 from the group consisting of antibody, lipid, protein, peptide, carbohydrate, nucleotides which contain or are derivatized to contain one or more of an amino, sulphhydryl, carbonyl, hydroxyl and carboxyl and thiophosphate groups, and oxy or deoxy polynucleic acids which contain or are derivatized to contain one or more of an amino, sulphhydryl, carbonyl, hydroxyl, carboxyl and
 25 thiophosphate groups, microbial materials, drugs, hormones, cells, cell membranes and toxins.